# Identification of a Potential Mitotic Function for the Mammalian Nup50

A Senior Thesis

Presented in Partial Fulfillment of the Requirements for graduation *with research distinction* in Biology in the undergraduate colleges of The Ohio State University

by

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## ABSTRACT

Mitosis is a conserved process in which the genetic material, DNA, is equally segregated between two daughter cells. DNA is contained in the nucleus of the eukaryotic cell and surrounded by the nuclear envelope. Multi protein complexes known as the Nuclear Pore Complexes (NPCs) embed within the nuclear envelope and regulate the transport of molecules in and out of the nucleus. Surprisingly, in *Aspergillus nidulans*, the model system used in my study, a nuclear pore complex protein Nup2 undergoes a unique translocation to chromatin during mitosis and is essential for proper mitotic progression. Interestingly, the Nup2 homolog in higher eukaryotes, Nup50, undergoes the same translocation. Therefore, the purpose of this study is to test whether Nup50 can translocate onto chromatin in *Aspergillus nidulans* and complement the mitotic function of Nup2. In order to test this hypothesis, the Nup50 gene was integrated into *A. nidulans* using homologous recombination. Four way fusion PCR was used to generate a DNA cassette that contains the Nup50 gene fused to $EGFP^2$ marker and its expression under control of the inducible promoter *alcA*. Once Nup50 was introduced into *A. nidulans,* Nup2 was deleted in the background. So far, we have discovered that Nup50 is present in the nucleus at interphase and disperses throughout the cell during mitosis in the absence or presence of the *Aspergillus nidulans* Nup2. Results showed that Nup50 cannot complement Nup2's function perhaps because it is not located at either the NPCs of mitotic chromatin. Furthermore, mmNup50 is a much smaller protein than Nup2 and it lacks a specific domain which is present in Nup2, termed domain 4. Domain 4 has been shown to be sufficient and necessary for Nup2's location to the pore and DNA. This suggests that introducing domain 4 into mmNup50 might tether it to the

pore at interphase and mitotic chromatin. We plan to create a version of Nup50 that carries domain 4 and replace the endogenous An-Nup2 with this version. We can then ask whether this version of Nup50 can complement Nup2's function. This study may provide evidence that the novel essential mitotic function of the *A. nidulans* Nup2 is conserved in humans. Knowledge gained from these experiments might help us identify a potentially novel essential function of the mammalian Nucleoporin 50 in mitosis, which is important to understanding the initiation and progression of human diseases that can arise from defective mitosis such as cancer.

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I would like to thank my committee member, Dr. Chuck Daniels for his time and efforts.

I would like to thank current Osmani Lab members, especially Dr. Sarine Markossian for her willingness to assist and collaborate with me through my project.

## DEDICATION

This work is dedicated

to my parents, Sleiman and Juliette, to my sisters

Maria and Danielle and to my brother John.

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# CHAPTER I

# INTRODUCTION

## 1.1 Problem Statement

Cancer has inevitably affected the lives of countless people today. However, due to the complexity of this disease and the uncontrolled division of cancer cells the extensive research throughout past several decades has still not uncovered all the regulators and the pathways involved in cancer initiation and progression. With mutations ranging from substitutions, insertions and deletions of bases in genes that regulate mitosis, we can only try to begin to interpret the genomic abnormalities of cancer cells in the hopes of improving the diagnosis and therapy of the disease. Cancer is essentially a disease in which cells misregulate mitosis and thus are converted from normal to cancerous cells. For a cell, the decision to enter mitosis is one of the most critical and dynamic steps in the cell cycle. Therefore, this process is under strict control by a network of regulatory pathways known as checkpoints, which act as brakes restricting the progression of mitosis due to several factors. Cancer cells deviate from the normal cell cycle and undergo misregulated mitosis with altered mitotic brakes thus leading to excessive division.

During higher eukaryotic mitosis, dramatic rearrangements of nuclei occur to divide the genomic material (DNA) and segregate it into two daughter cells. As the cell enters mitosis, the nuclear pore complexes and the nuclear envelope that encloses the nucleus are disassembled while simultaneous chromosomal condensation and microtubule rearrangements takes place. Thus, remodeling of many architectural features of the cell is a prerequisite for cell division.

Interesting and recent evidence shows that the nuclear pore complex proteins play critical roles in orchestrating these events. It has been shown that in *Aspergillus nidulans*, the genetic model organism used in my research, one nuclear pore complex protein Nup2 undergoes a unique translocation to DNA during mitosis and is essential for proper mitotic progression (Osmani et al., 2006). Interestingly, the Nup2 homolog in higher eukaryotes, Nup50, undergoes the same translocation (Dultz et al., 2008). The purpose of this study then is to use this fungus as an inroad into understanding how the pore contributes mechanistically to cell division in higher eukaryotes. In particular, I will test whether Nup50 can translocate onto chromatin in *Aspergillus nidulans* and complement the mitotic function of Nup2. This work might help us identify a potentially novel essential function of the mammalian Nucleoporin 50 in mitosis. These will then progress our understanding in fundamental aspects of the cell division in hopes of ultimately lead us to some sort of understanding of the initiation and progression of human diseases that can arise from defective mitosis such as cancer.

### 1.2 Biology of *Aspergillus nidulans*

*Aspergillus nidulans* continues to be an important model organisms used for research in cell biology today. It is a non-pathogenic species with a genome of 31Mb that has been entirely sequenced along with thousands of mapped genes (Galagan, 2008). *Aspergillus nidulans*, (teleomorph Emericella nidulans) is a filamentous fungus of the phylum Ascomycota that undergoes sexual, asexual and parasexual life cycles. It is mainly haploid but can also form heterokaryons which are hyphae containing two different types of nuclei in one cytoplasm. Heterokaryons can form by hyphal fusion, on the other hand, vegetative diploids form by nuclear fusion (karyogamy). *Aspergillus nidulans* undergoes parasynchronous mitosis. Nuclei of this fungus divide several times without cytokinesis. Usually, up to eight nuclei can share a common

cytoplasm until a septum forms. This fungus displays a rapid growth rate at about 0.5 mm per hour at 37°C and has a cell cycle of about 100min consisting of a G1 phase of 15 min, an S phase of 40 min, a clearly defined G2 phase of 40min, and a mitotic phase (M phase) of only 5 min, under ideal growth conditions (Oakley, 1993). In this study we utilized the asexual cycle of *Aspergillus nidulans* to perform our studies. In the asexual cycle, a haploid uninucleate conidiospore undergoes isotropic growth followed by polarized growth which leads to the formation of the germtube. This fungus then forms vegetative hyphae which then differentiate to give a fruiting body termed the conidiophore. Conidiophores usually generate multiple conidiospores, asexual spores (Casselton and Zolan, 2002; Todd et al., 2007a, b). The asexual cycle is ideal for gene targeting and replacement experiments and is an ideal place to follow protein location dynamics throughout the cell cycle by live cell confocal microscopy. Because *A. nidulans* is a well characterized and established model genetic system that has many distinct and useful qualities, I was interested to conduct my research using this fungus.

**1.3 The Cell Cycle**

Cells need to multiply in order to survive. The cycle of cellular division is termed the cell cycle. It is an essential process that ensures the continuity of all life. Unicellular organisms propagate by cell division. Multicellular organisms rely on cell division during development and tissue repair. Moreover, proper regulation of the cell cycle ensures proper segregation of the genetic material from mother to daughter cells in a timely and regulated manner thus contributing to the fundamental aspects of a healthy organism. Any misregulation of cell division leads to the initiation and progression of cancer and can in turn lead to birth defects and lethality. The eukaryotic cell cycle is divided into interphase and M-phase. During interphase the

cells grow in size as they prepare for division, accumulate nutrients and replicate their genome. These processes are allocated into separate phases: G1, where cells grow and accumulate nutrients, S, where chromosomes duplicate and G2, where cells mature and decide whether to enter into mitosis. In mitosis, cells segregate their genetic material, the DNA, into two daughter nuclei. Nuclear division, in which separation of the chromosomes occur is termed mitosis. Mitosis is then followed by the partitioning of the cytoplasm (cytokinesis) which results in two separate daughter cells. Like interphase, mitosis is also divided into separate phases. First, during prophase, the chromosomes condense, the mammalian NPCs disassemble and the nuclear envelope breaks down. This is subsequently followed by metaphase where the chromosomes align to the metaphase plate and the mitotic spindle is formed. Next, during anaphase the mitotic spindle separates the daughter chromatids. This phase is then followed by telophase where the spindle disassembles and the cytoplasm partitions forming 2 separate daughter cells.

Primarily, the most imperative role of the cell division cycle is the faithful maintenance and transmission of the genetic material. Cells have evolved distinct mechanisms to ensure proper progression of events in a consecutive manner during its cycle. These mechanisms are termed checkpoints. These checkpoints usually occur during transition from one phase to another and ensure that one cell cycle phase is properly completed before transitioning into the following phase. Specifically, the checkpoint between the $G_2$ to M phase prevents cells that have not undergone DNA replication and damage repair from continuing into the next phase. Furthermore, the spindle assembly checkpoint (SAC) during mitosis prevents cells from entering anaphase until sister chromatids are aligned along the metaphase plate with correct bipolar spindle attachments. Consequently, over the entire course of cellular growth and division, genetic information is preserved by means of checkpoints (Alberts, 2008).

## 1.4 Nuclear Pore Complexes

Multi protein complexes known as the Nuclear Pore Complexes (NPCs) embed the nuclear envelope and regulate the transport of molecules in and out of the nucleus. They are made of about 30 nucleoporins, commonly abbreviated as Nups. These Nups are present as multiple copies in each pore. Free diffusion of small molecules (usually smaller than 40kD) occurs through these pores along with active transport of larger proteins and nucleic acids. The NPC structure is largely conserved from lower to higher eukaryotes and is comprised of core and peripheral Nups. Some peripheral Nups reside at the nuclear side and form the nuclear basket whereas others are more specific to the cytoplasmic side and form the cytoplasmic fibrils (Alber et al., 2007; D'Angelo and Hetzer, 2008; Stewart, 2007).

## 1.5 Regulation of Nuclear Pore complexes in mitosis

In higher eukaryotes, the process of mitosis is characterized as open. During open mitosis, the NPCs fully disassemble and the nuclear envelope completely breaks down. This is then followed by a stepwise reassembly of the NPCs, reforming of the NE along with chromosomal segregation upon mitotic exit. Conversely, in some lower eukaryotes, mitosis is termed closed. In closed mitosis, the NPCs and the Nuclear envelope stay intact during mitosis and the nucleus is usually separated by a single pinch of the nuclear envelope (Guttinger et al., 2009). Interestingly, in the model organism *Aspergillus nidulans*, the process of mitosis is thought to be an intermediate of the former two processes and is accordingly termed semi-open. This is because, the NPCs partially disassemble in this fungus, and the NE seems to stay intact although it becomes semi-permeable. During the semi-open mitosis of *Aspergillus nidulans*,

proteins can freely access the nucleus through passive diffusion (Figure 1.1) (De Souza et al., 2004; De Souza and Osmani, 2007, 2009). This dynamic disassembly and reassembly of NPCs during the open and semi-open mitosis is shown to be under the regulation of many mitotic kinases, such as the NIMA kinase and the CDK1 kinase (De Souza and Osmani, 2009; Laurell et al., 2011). Significantly, data from recent years have come to show that many of the Nups that leave the pore during mitosis move to distinct locations during the process where they exhibit essential mitotic roles (Guttinger et al., 2009).

**1.6 Nucleoporin 2 and its regulation during mitosis**

The nuclear pore complex protein Nup2 is a peripheral nuclear basket Nup which holds a highly conserved overall domain structure spanning from lower to higher eukaryotes. The Nup2 protein carries multiple domains, an N-terminal importin-α binding domain, a domain rich in FG repeats, and a C-terminal Ran GTPase binding domain (Figure 1.2). The *Aspergillus nidulans* Nup2, is a significantly larger protein than its yeast and human counterparts. The proposed reason as to why An-Nup2 is a much larger protein can be attributed to the idea that it carries additional functional domains not present in the other proteins. Studies in yeast and mammals have shown that Nup2 carries a role in helping the disassembly of the importin α-β/cargo complexes within the nucleus by binding to Ran GTP and bringing it close to the complex (Stewart, 2007) . Thus it has a nonessential role in nuclear import at interphase which is to make the import more efficient.

During the *Aspergillus nidulans* mitosis, Nup2 undergoes a unique translocation from the pore to DNA during mitosis (Osmani et al., 2006) and has been recently shown to be essential for proper mitotic progression (Markossian, unpublished). Interestingly, the Nup2 homolog in

higher eukaryotes, Nup50, undergoes the same translocation (Dultz et al., 2008). This is very intriguing and raises the question whether this essential mitotic function of Nup2 is conserved from lower to higher eukaryotes.

### 1.7 Hypothesis

According to the fact that the mammalian Nup2 translocates to DNA during mitosis we propose that it would complement the essential mitotic role of Nup2 on chromatin in *Aspergillus nidulans*. We plan to introduce the mammalian Nup50 to Aspergillus nidulans and study whether it complements An-Nup2's function. This study then will investigate whether the novel essential mitotic function of *A. nidulans* Nup2 is conserved in humans.

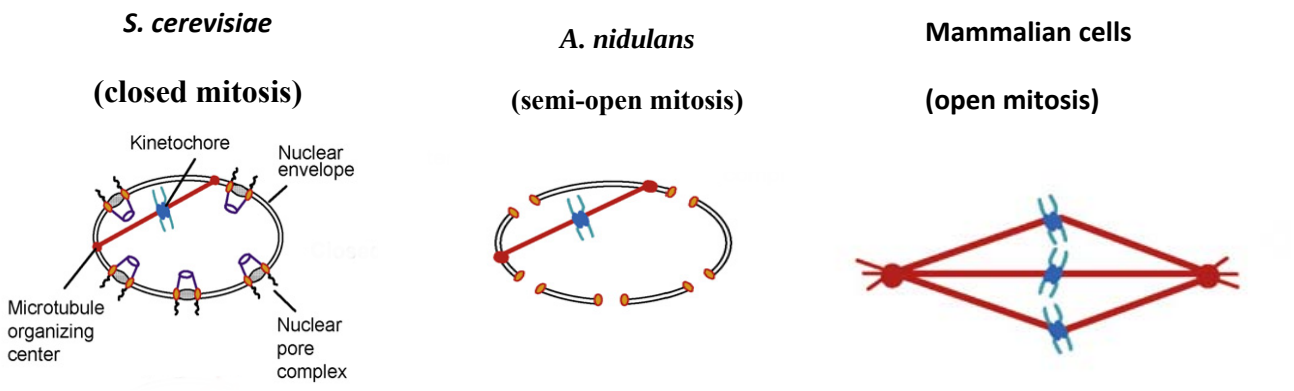



**Figure 1.1:** *A. nidulans* undergoes a semi-open mitosis, in which the nuclear envelope stays intact but the NPC partially disassembles (modified from C.P. De Souza and S.A. Osmani, Trends in Genetics, 2009).

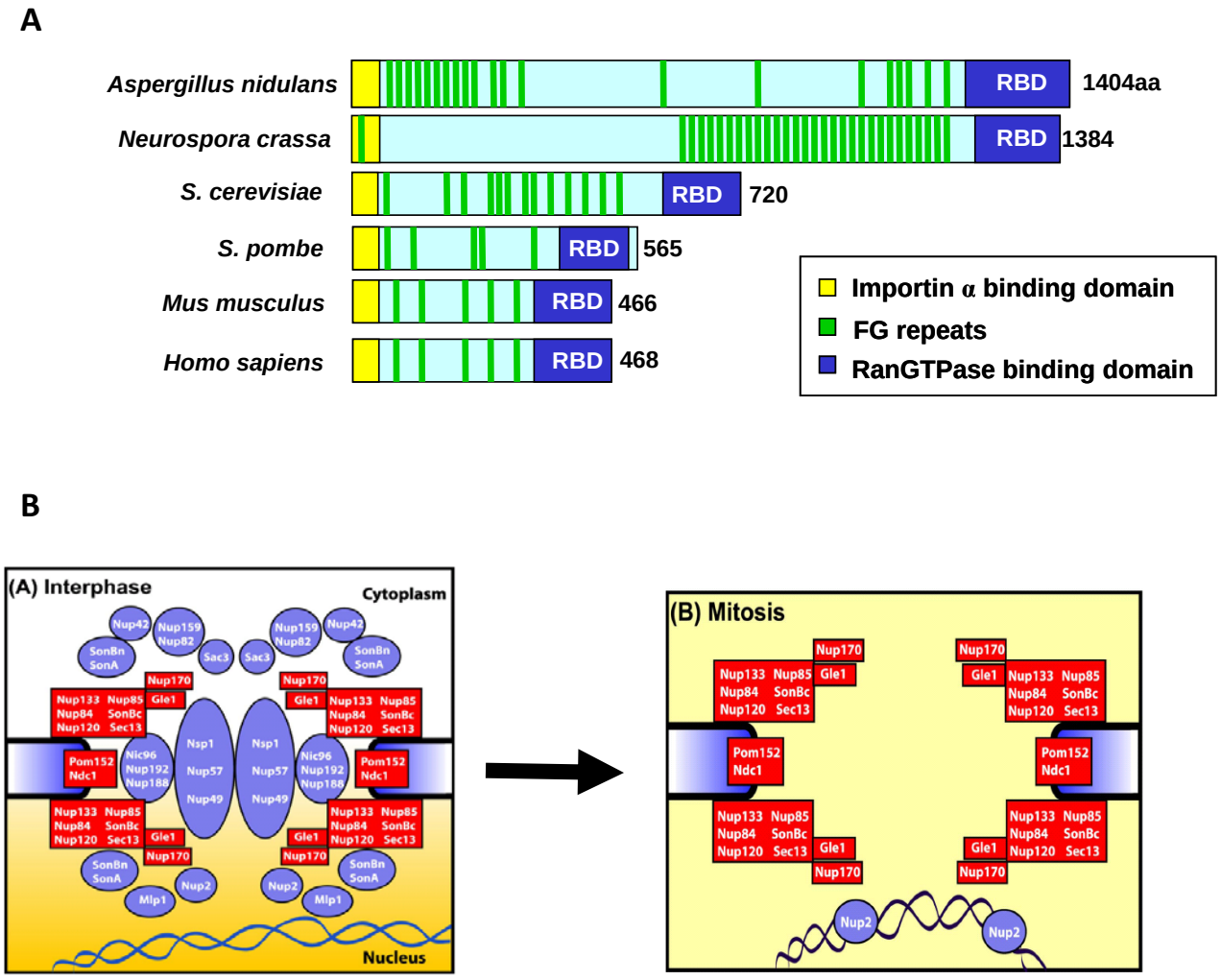



**Figure 1.2:** (**A**) Depiction of different orthologs and domain structure of Nup2 showing that the *A. nidulans, M. musculus* and *H. sapiens* Nup2 contain the same specific domains. (**B**) Cartoon depicting the NPC of *Aspergillus nidulans* at interphase and mitosis. While peripheral Nups (in blue) disperse from the NPC in mitosis, the core Nups (in red) remain associated with the NPC. Specifically, one of these nucleoporins, Nup2 moves to chromatin during mitosis. It remains on chromatin during mitosis until the G1 phase where it returns to the NPC. (modified from Osmani *et al.,* Molecular Biology of the Cell, 2006).

# CHAPTER II

# MATERIALS AND METHODS

## 2.1 Oligonucleotide Primer Selection and Polymerase Chain Reaction (PCR)

The oligonucleotide primers were specifically designed to have sequences complimentary to the ends of the DNA segments of interest. This complementation allows for hybridization of the primers to the template strand during PCR. Primers with a greater percentage of guanine-cytosine pairs, around 50-60 %, allow for a higher affinity between the primer and template. This can be attributed to the fact that guanine and cytosine have three hydrogen bonds that occur between this nucleotide base pair, versus the two hydrogen bonds that keep adenine and thymine together. This was done using MS-DOS primer software. The primer sequences were then sent to either Integrated DNA Technologies or Sigma Aldrich where they were custom-made.

To amplify the region of interest, PCR was conducted using as template *A. nidulans* genomic DNA or plasmids containing the appropriate cassettes according to Roche Expand Long Template PCR System. PCR involves denaturation of the double-stranded DNA into single strands at ~ 94 °C. Next, the temperature is decreased to 50-60 °C, allowing the specifically designed primers to anneal to their complimentary sequences, which are located at the ends of the desired amplified segment. The temperature is then increased to ~ 68 ° C so that the polymerase enzyme extends the primers using added dNTP's and therefore leading to the synthesis of DNA from 5' to 3' direction. The entire cycle is then repeated after the temperature is raised to ~ 94 °C again. Over time, the desired segment is amplified exponentially. Temperatures, number of cycles and other conditions were optimized according to the specific

reaction. In this project PCR was done using a 9700 Thermal Cycler (Perkin Elmer) or a Gradient Thermal Cycler (Eppendorf).

**2.2 Agarose Gel Electrophoresis**

PCR products were run on a 0.8% agarose gel for verification and analysis. The negatively charged DNA is loaded into the wells and Ethidium bromide (Eb) is added to the running buffer. Eb intercalates into DNA and fluoresces allowing visualization of the bands under UV light. An electrical current is then applied to the gel which causes the DNA to migrate from the negative pole towards the positive. Larger fragments migrated at a slower rate through the gel in comparison to smaller ones. Lambda Hind III ladder was also run on the gel as a marker to quantify and verify the size of the bands.

**2.3 Specific media for culture**

YG media: (56 mM dextrose, 5 g/L yeast extract, 10 mM magnesium sulfate, supplemented with 1 μg/ml p-aminobenzoic acid (paba), 0.5 μg/ml pyrodoxine HCL (pyro), 2.5 μg/ml riboflavin HCL (ribo), 2 μg/ml nicotinic acid, 20 μg/ml choline, 20ng/ml D-biotin and 1 ml/l trace elements). Strains carrying the *pryG89* auxotrphic mutation were grown in YGUU (YG media supplemented with 1.2 g/l uridine and 1.12 g/l uracil).

YAG media: (YG media with 15g/L agar).

MAG media: (20 g/l malt extract, 20 g/l bacto peptone, 56 mM dextrose, supplemented with 1 μg/ml p-aminobenzoic acid (paba), 0.5 μg/ml pyrodoxine HCL (pyro), 2.5 μg/ml riboflavin HCL (ribo), 2 μg/ml nicotinic acid , 20 μg/ml choline, 20 ng/ml D-biotin, 50 mg/l adenine sulfate, 50 mg/l leucine, 50 mg/l L-methionine, 100 mg/l arginine, 200 mg/l L-lysine HCL, 1 ml/l trace

elements and 2% agar). Strains carrying uncomplemented *pryG89* auxotrphic mutation were grown on MAGUU (MAG media supplemented with 1.2 g/l uridine and 1.12 g/l uracil).

Minimal Media Urea: (10 mM urea, 7 mM potassium chloride, 1 mM magnesium sulfate, 1 ml/l trace elements, and supplements as required). Glucose (final concentration 1% w/v) or glycerol (finaconcentaration 0.47 % v/v) was added prior to autoclaving. Ethanol (final concentration 1% v/v) was added after autoclaving. In addition, potassium phosphate [pH 6.8] (12 mM) and sodium thiosulfate (3.2 mM) were added after autoclaving. For solid media 1.5% w/v agar was added prior to autoclaving.

**2.4 Preparation of A. nidulans conidia stock suspensions**

*A. nidulans* conidiospores were inoculated into 4mL of MAG or MAGUU top agar media containing only 0.75% agar at 48°C. This media is then vortexed and overlayed onto MAG or MAGUU plates. These plates were incubated at 32°C for 30 to 40 hours until conidia were ready to harvest. Fresh conidiospores were harvested in 10mL of 0.2% Tween 20 using a sterile glass spreader. Suspended conidia were transferred to sterile 15mL falcon tubes (Corning). The suspensions were centrifuged for 2 minutes to sediment the conidiospores. Hyphal debris was removed by gently resuspending and recovering only the top conidial layer of the pellet. The collected conidia were washed two times in 10mL of 0.2% Tween 20. After the final wash the conidia were resuspended in stock storage solution (8.5mM sodium chloride, 200μM Tween 80). Concentration of conidiospores in suspension were quantified by counting 10 μl of a 1 x $10^{-3}$ dilution of conidia in 0.2% Tween 20 using a Bright-Line hemocytometer (Reichert-Jung). Three fields of conidia were counted for each sample, and the average value was used for

quantification. The number of conidia obtained from this count was multiplied by 1 x $10^7$ to determine the concentration of the original suspension in spores/mL.

**2.5 Long term storage and stock preparation of A. nidulans**

A single colony was replica plated on a selective media plate using a sterile toothpick and incubated at 32°C for 48 hours followed by 3 to 5 days at room temperature. 5mL of sterile 7.5% milk (7.5g of Carnation Nonfat Dry Milk in 100mLs dH2O and autoclaved for 20 minutes) was added onto the plate and conidiospores were harvested by rubbing the top of the fungal lawn to release conidia into suspension using a sterile glass spreader. Then, 250µl of the suspension was transferred into each of two glass vials containing baked, sterile silica and placed on ice for one hour. The silica was then vortexed to evenly distribute the spores and returned to ice for an additonal 30 minutes. Subsequently, the silica was left at room temperature for 2 to 3 days with loosened lids to promote complete drying. After 3 days, the silica was again vortexed and then the vials placed in a room temperature desiccator. Strains were re-grown when needed from silica stocks by placing 5-10 silica pieces onto appropriate solid media plates, and incubating at 32°C for several days in an air incubator.

**2.6 Transformation**

1 x $10^9$ fresh conidia were inoculated into 50 mL YGUU, and grown at 32°C in an air incubator with 200rpm for about 6 hours or until conidia began to germinate. Germlings were harvested by centrifugation for 2 minutes and resuspended in a protoplasting mix containing 20 mL Solution 1 (105.6 g/L ammonium sulfate, 19.2 g/L citric acid, [pH 6.0]), 20 mL Solution 2 (10 g/L yeast extract, 20 g/L sucrose, 1 µg/mL acid paba, 0.5 µg/mL pyro, 2.5 µg/mL ribo, trace elements, 4.92 g/L MgSO4), 80 mg bovine serum albumin (BSA), 10 mg/ml of the enzyme

VINOFLOW FC. The resuspended germlings were transferred to a clean sterile flask, and incubated in an air incubator at 32°C for 2 to 3 hours until protoplasts were formed. Protoplasts were distinguished by their visible large vacuoles under a light microscope. The protoplasts were then centrifuged for 2 minutes at 2,000 rpm, and washed twice with Solution 3 (52.8 g/L ammonium sulfate, 10 g/L sucrose, 9.6 g/L citric acid, [pH 6.0]), then resuspended in 1 mL Solution 5 (44.7 g/L KCl, 7.35 g/L CaCl2, 2.09 g/L MOPS, [pH 6.0]). Transformation was then followed by combining 2-4 µg DNA, 100µL protoplasts, and 50 µL of room temperature Solution 4 (250 g/L PEG 8000, 7.35 g/L CaCl2, 44.7 g/L KCl, 10 mL 1 M Tris [pH 7.5]). The transformation reaction was first incubated on ice for 20 minutes, then an additional 1mL of Solution 4 was added to the mix and incubated at room temperature for another 20 minutes. If the selection marker was the auxotrophic mutation *pyrG89*, then aliquots of 10 µL, 25µL, 50 µL, 100 µL, 250 µL, and 500 µL volumes of the transformation mix was added in 4 mL YAG sucrose and overlayed onto YAG sucrose plates. If the selection marker was *pyroA4*, then different volumes of the transformation mix were added to 4mL mmUrea sucrose and plated onto mmUrea sucrose plates. Transformation plates were incubated in an incubator at 32°C for about 70 hours or until colonies emerged. Transformations were conducted in *nKu*A$^{\boldsymbol{ku70}}\Delta$ strains (mainly SO451) which have been shown to have a very high frequency of homologous gene targeting (Nayak et al., 2006).

**2.7 Counter selection on 5-FOA**

Counterselection on 5-FOA was performed when selecting for transformants based on the absence of the wild type *pyrG* gene. The generated DNA cassette was prepared targeting the *pyrG* locus. After transformation, the transformation mix was added to top agar containing YAGUU with sucrose, and transferred into empty Petri dishes. These plates were then left

overnight at room temperature. Then the plates were autoclaved and overlaid with YAGUU + 1mg/ml 5-FOA (5FOA (1mg/ml 5FOA). The plates were then incubated at 32 ºC for 3 - 5 days for transformants to emerge.

**2.8 Genomic DNA extraction**

We used small scale extractions to isolate *Aspergillus nidulans* genomic DNA for diagnostic PCRs. Accordingly, a small amount of spores were inoculated in petri dishes with appropriate media. Mycelia was then harvested by vacuum filtration of the media through Miracloth (Calbiochem), and washed twice with cold Stop Buffer (9 g/L sodium chloride, 65 mg/L sodium azide, 20 mL 0.5 M EDTA [pH 8.0], 2.1 g/L sodium fluoride). Excess liquid was pressed out of mycelia which were placed in an eppendorf, and immediately immersed in liquid nitrogen. The mycelia were then removed from the liquid nitrogen and lyophilized overnight. The dried mycelia were crushed, and then 100 µl of Miniprep Lysis Solution (Promega) was added to it and vortexed to mix. 100 µl Miniprep Neutralization Solution was then added and vortexed to mix. Samples were centrifuged at 14,000 rpm in a Model 5420 table top refrigerated centrifuge (Eppendorf) for 10 minutes. The supernatant was processed to isolate the DNA using a Miniprep Purification Kit (Promega). Genomic DNA was eluted from the column in 50 µl $dH_2O$.

**2.9 *alcA* driven protein expression**

*alcA* driven protein expression was performed either in liquid cultures for protein preparation or on solid media for phenotypic analysis. Cells were grown either in minimal media with 1% ethanol (inducing conditions) or 0.47 % glycerol (non-repressive/non-inductive conditions). Minimal media with 1% dextrose was used for repressing the promoter.

## 2.10 Heterokaryon rescue technique for the identification and analysis of essential genes in Aspergillus nidulans

The heterokaryon rescue technique was generated in our laboratory to positively identify essential genes. Heterokaryons were generated by transforming *pyrG89* recipient strains carrying *nku*A$^{ku70}$Δ (Nayak et al., 2006), with ΔNup2::pyrG$^{Af}$ linear constructs. Heterokaryons, which produce two types of uninucleate conidia, were identified by streaking transformants on rich YAG media with or without Uridine and Uracil. Only ΔNup2::pyrG$^{Af}$ could germinate in the absence of uridine and uracil (UU). Media lacking UU was used to study the phenotypes of Nup2. These heterokaryons were propagated by cutting a small portion of mycelia from the growing edge of a colony, and placing it onto the middle of a YAG plate.

## 2.11 Live cell imaging

In order to examine fluorescently tagged proteins in living cells, cells were germinated in glass bottom Petri dishes (MatTek Cultureware). Wild type haploid strains were germinated in 3 ml of minimal media at room temperature (21-23°C) for 24 hours or until the 4 to 8 nuclear stage was reached. For *nup2* null mutant analysis, spores from heterokaryons were germinated at room temperature in YG medium with or without UU for about 8 hours or overnight depending on the projected stage of mitosis needed to analyze. Visualization of GFP/chRed fusion proteins was performed using inverted microscopes (Nikon) equipped for spinning disc confocal microscopy at room temperature.

## 2.12 Generation and analysis of strains carrying alcA-EGFP2-Nup50

EGFP2-mmNup50 was introduced under the alcohol dehydrogenase inducible promoter *alcA* at the *pyrG* locus of *Aspergillus nidulans* by homologous recombination (Figure 2.1 and 3.1

A). Four way fusion PCR was used to prepare the *alcA::EGFP2-mmNup50* cassette which is targeted to the *pyrG* locus. This cassette was then transformed in strain SO455, and transformants were selected on 5FOA+UU. Live cell imaging was performed in order to follow EGFP2-Nup50 throughout the cell cycle in *Aspergillus nidulans*. The spores carrying the cassette were grown in 3 ml of minimal media in the presence of 1% Ethanol and UU to induce protein expression. *alcA* driven protein expression was performed either in liquid cultures for protein preparation or on solid media for phenotypic analysis. Moreover, minimal media with dextrose was used for repressing the promotor.

**2.13 Complementation Study**

Nup2 was deleted in strains carrying *alcA::EGFP2-mmNup50* (Figure 2.2). The transformants were grown on YAG (Yeast extract glucose) plates with 1 M sucrose. To test for heterokaryons, spores from different transformants were streaked on both YAGUU and YAG plates. Heterokaryons contain two genetically distinct type of nuclei in one cytoplasm and are usually generated due to the deletion of essential genes. One nucleus has the functional pyrG allele and lacks Nup2, the other has the wild-type Nup2 allele but lacks pyrG. Heterokaryons were isolated since many of the transformants grew on YAGUU but not on YAG. The two transformants that did grow on YAG probably are diploid cells. Cell growth was assessed after 1 or 3 days at 32°C.

## 2.14 Endogenous replacement of Nup2 with Nup50 with or without domain 4

EGFP2-Nup50, D4::EGFP2-Nup50, EGFP2-Nup50::D4 proteins will be expressed in *A. nidulans* under the endogenous Nup2 promoter. Three Constructs *EGFP2-mmNup50::pyrG* **,** *EGFP2-mmNup50::D4::pyrG* , *D4::EGFP2-mmNup50::pyrG* were made to target and replace the *nup2* locus by homologous recombination (Figure 2.3, 2.4 and 2.5). For further details refer to the figure legends. These cassettes will then be transformed into strain SO451 (*pyrG89*) and transformants will be selected on –UU. Transformants will be then streaked on YAG plates with or without UU to assess whether they are heterokaryons (expressed protein does not complement *Δnup2*) or haploid (expressed protein does complement *Δnup2*).

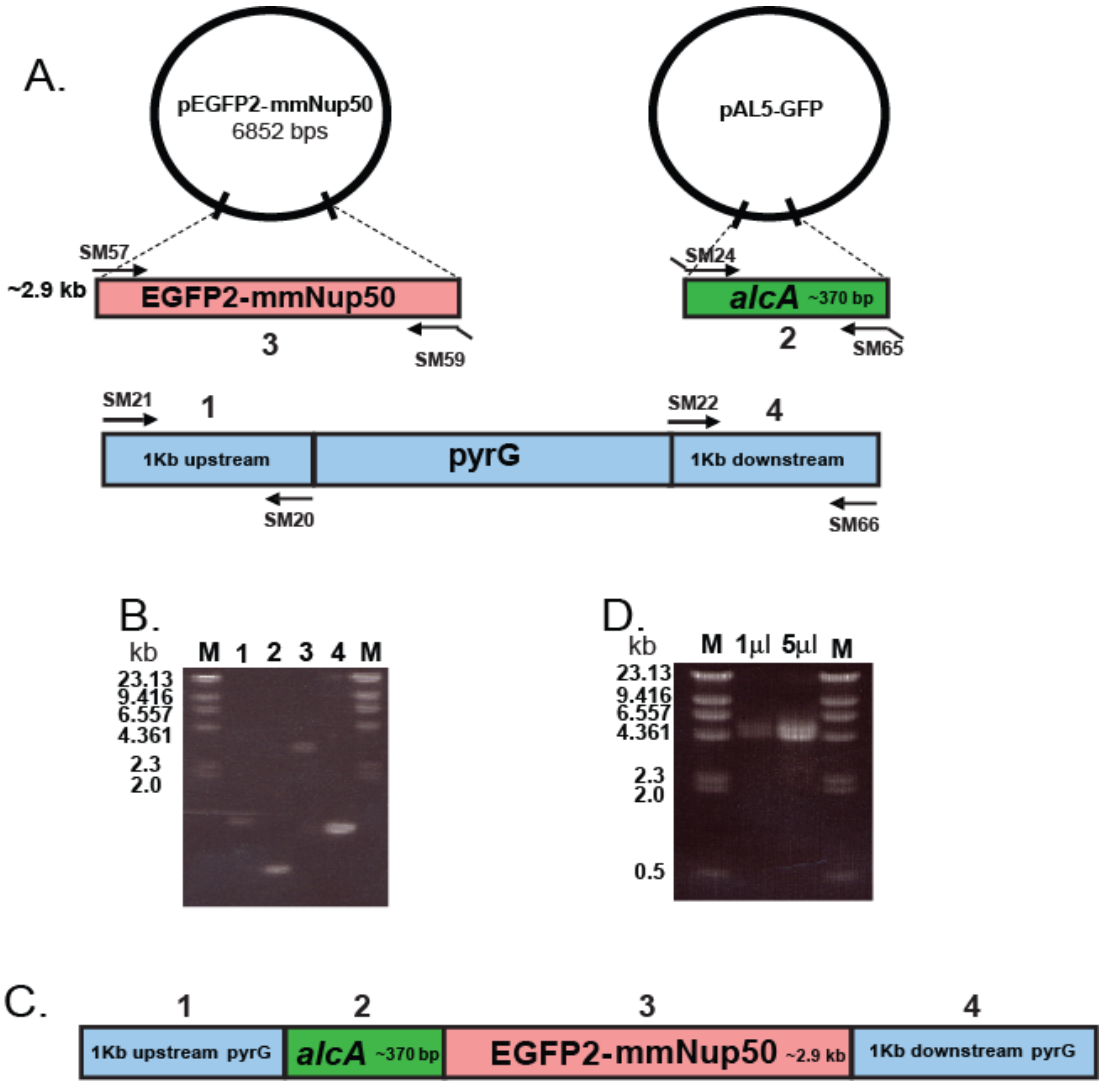



**Figure 2.1 Generation of EGFP2-*alc*A-mmNup50 cassette targeted to the *pyrG* locus of *Aspergillus nidulans*. (A)** mmNup50 cDNA tagged with two EGFPs (piece 2) was amplified by primers SM57/SM59 from pEGFP2-mmNup50 (gift from J. Ellenberg, EMBL, Heidelberg, Germany.) *alcA* was amplified by primers SM24/SM65 from the plasmid pAL5-GFP (piece 2), and 1KB upstream (piece 1) and downstream (piece 4) of *pyrG* were amplified with primer pairs SM21/20 and SM22/66 respectively. **(B)** Agarose gel showing PCR products of individual pieces used to generate the fused cassette. **(C)** A cartoon depicting the cassette made by 4-way fusion PCR of the pieces 1 to 4. **(D)** One and five micro liters of the fusion PCR product were run on a 0.8% agarose gel. This experiment was performed in collaboration with Dr. Sarine Markossian.

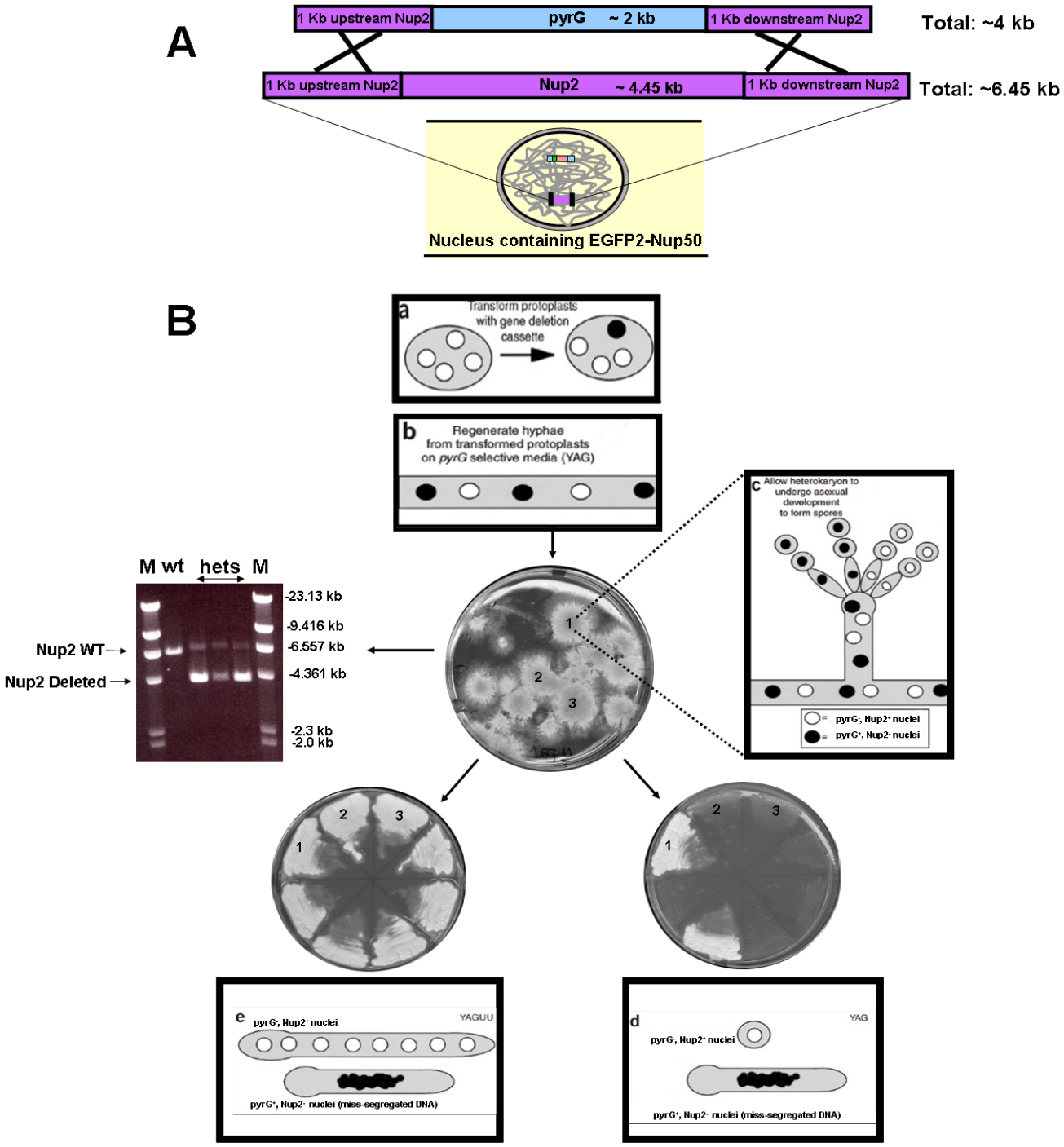

A
1 Kb upstream Nup2
pyrG ~ 2 kb
1 Kb downstream Nup2
Total: ~4 kb
1 Kb upstream Nup2
Nup2 ~ 4.45 kb
1 Kb downstream Nup2
Total: ~6.45 kb
Nucleus containing EGFP2-Nup50
B
a
Transform protoplasts with gene deletion cassette
b
Regenerate hyphae from transformed protoplasts on pyrG selective media (YAG)
c
Allow heterokaryon to undergo asexual development to form spores
pyrG-, Nup2+ nuclei
pyrG+, Nup2- nuclei
M wt hets M
-23.13 kb
-9.416 kb
-6.557 kb
-4.361 kb
-2.3 kb
-2.0 kb
Nup2 WT
Nup2 Deleted
1
2
3
e
pyrG-, Nup2+ nuclei
YAGUU
pyrG+, Nup2- nuclei (miss-segregated DNA)
d
pyrG-, Nup2+ nuclei
YAG
pyrG+, Nup2- nuclei (miss-segregated DNA)

**Figure 2.2. Deletion of Nup2 in the *Aspergillus nidulans* strain carrying the EGFP2-*alc*A-mmNup50 using the heterokaryon rescue technique. (A)** The pyrG gene was targeted to the Nup2 locus by homologous recombination. The Nup2 deletion cassette was generated by 3-way fusion PCR. **(B)** The transformants were grown on YAG (Yeast extract glucose) plates with 1 M sucrose. To test for heterokaryons, spores from different transformants were streaked on both YAGUU and YAG plates. Heterokaryons contain two genetically distinct type of nuclei in one cytoplasm and are usually generated due to the deletion of essential genes. One nucleus has the functional pyrG allele and lacks Nup2, the other has the wild-type Nup2 allele but lacks pyrG. Heterokaryons were isolated since many of the transformants grew on YAGUU but not on YAG. The two transformants that did grow on YAG probably are diploid cells.

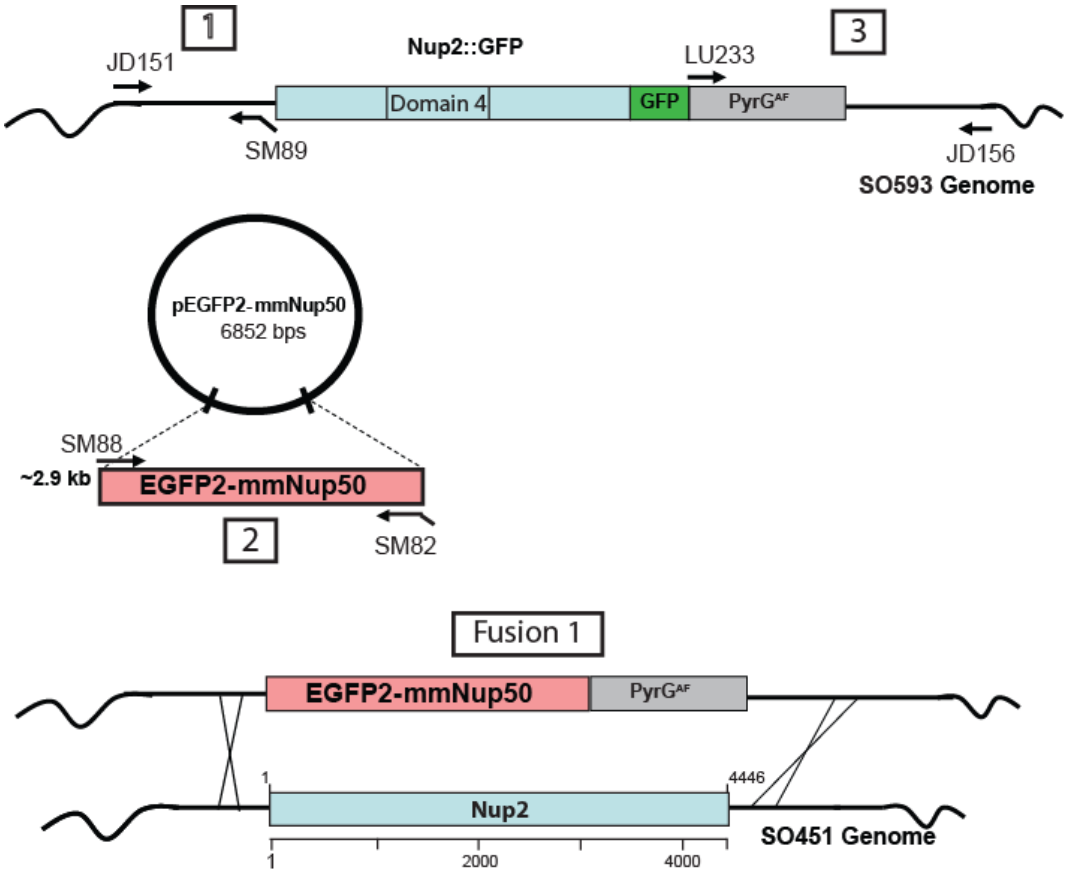
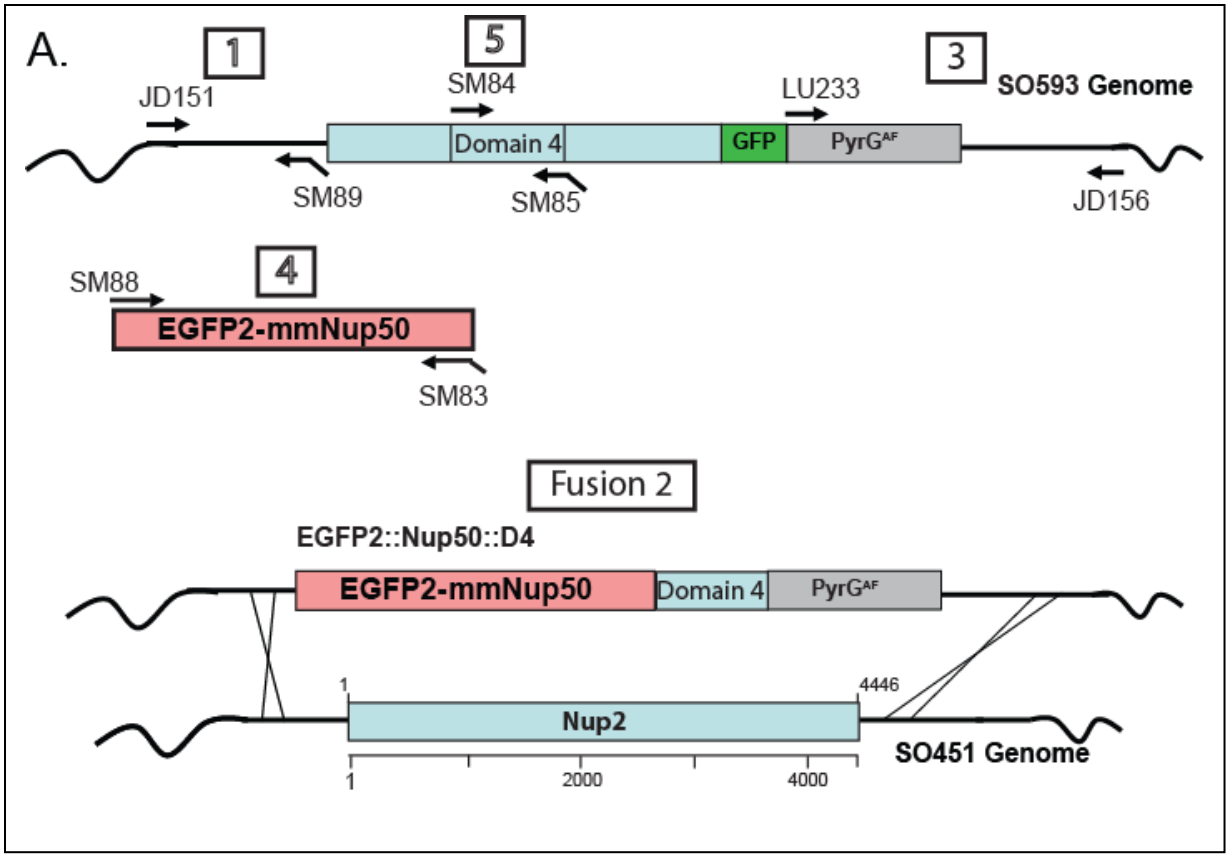



**Figure 2.3 Replacement of endogenous *A. nidulans* Nup2 with EGFP2-mmNup50.** *EGFP2-mmNup50::PyrG* will be placed under the endogenous *nup2* promotor in the *A. nidulans* genome. To accomplish that, a Nup2 deletion cassette is made that targets the *nup2* locus, and replaces the endogenous *nup2* in the genome with *EGFP2-mmNup50::PyrG*. 1kb upstream of Nup2 is amplified with primers JD151/SM89; then another piece (carrying Pyrg + 1kb downstream of Nup2) is amplified using the primers LU233 and JD156 from the genome of SO593, whose *nup2* locus was previously tagged with GFP::PyrG. EGFP2-mmNup50 is amplified using primers SM88 and SM82. 3-way fusion PCR of these three pieces will create the *EGFP2-mmNup50::PyrG* cassette. This cassette will be then transformed in SO451 (*pyrG89*) and transformants will be selected on –UU. This experiment is being conducted in collaboration with Dr. Sarine Markossian.


A.
1
JD151
5
SM84
3
SO593 Genome
LU233
Domain 4
GFP
PyrG^AF
SM89
SM85
JD156
SM88
4
EGFP2-mmNup50
SM83
Fusion 2
EGFP2::Nup50::D4
EGFP2-mmNup50
Domain 4
PyrG^AF
1
4446
Nup2
SO451 Genome
1
2000
4000

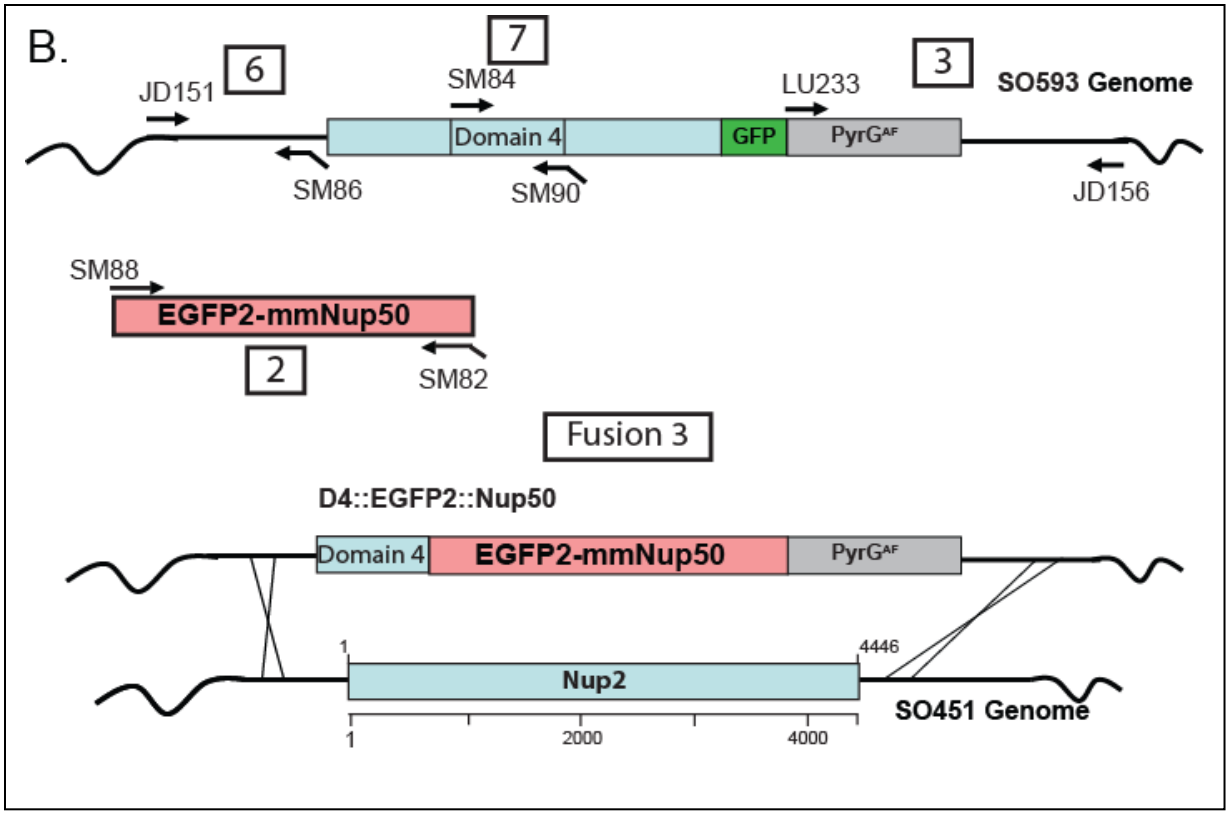

B.
6
JD151
7
SM84
3
SO593 Genome
LU233
Domain 4
GFP
PyrG^AF
SM86
SM90
JD156
SM88
EGFP2-mmNup50
2
SM82
Fusion 3
D4::EGFP2::Nup50
Domain 4
EGFP2-mmNup50
PyrG^AF
1
4446
Nup2
SO451 Genome
1
2000
4000

**Figure 2.4: Replacement of endogenous *A. nidulans* Nup2 with EGFP2-mmNup50 containing domain 4.** (A-B) *EGFP2-mmNup50::PyrG* containing domain 4 will be placed under the endogenous *nup2* promotor in the *A. nidulans* genome. To accomplish that, two different Nup2 deletion cassette are made that target the *nup2* locus, and replace the endogenous *nup2* in the genome with either *EGFP2-mmNup50::D4::PyrG* (A) or *D4::EGFP2-mmNup50::PyrG* (B). 1kb upstream of Nup2 is amplified with either primers JD151/SM89 or JD151/SM86; then another piece (carrying PyrG + 1kb downstream of Nup2) is amplified using the primers LU233 and JD156 from the genome of SO593, whose *nup2* locus was previously tagged with GFP::PyrG. EGFP2-mmNup50 is amplified using primers either SM88/SM83 or SM88/SM82. Domain 4 is amplified using primers either SM84/SM85 or SM84/SM90. 4-way fusion PCR of the specific four pieces will create the individual cassettes. These cassettes will be then transformed in SO451 (*pyrG89*) and transformants will be selected on –UU. This experiment is being conducted in collaboration with Dr. Sarine Markossian.

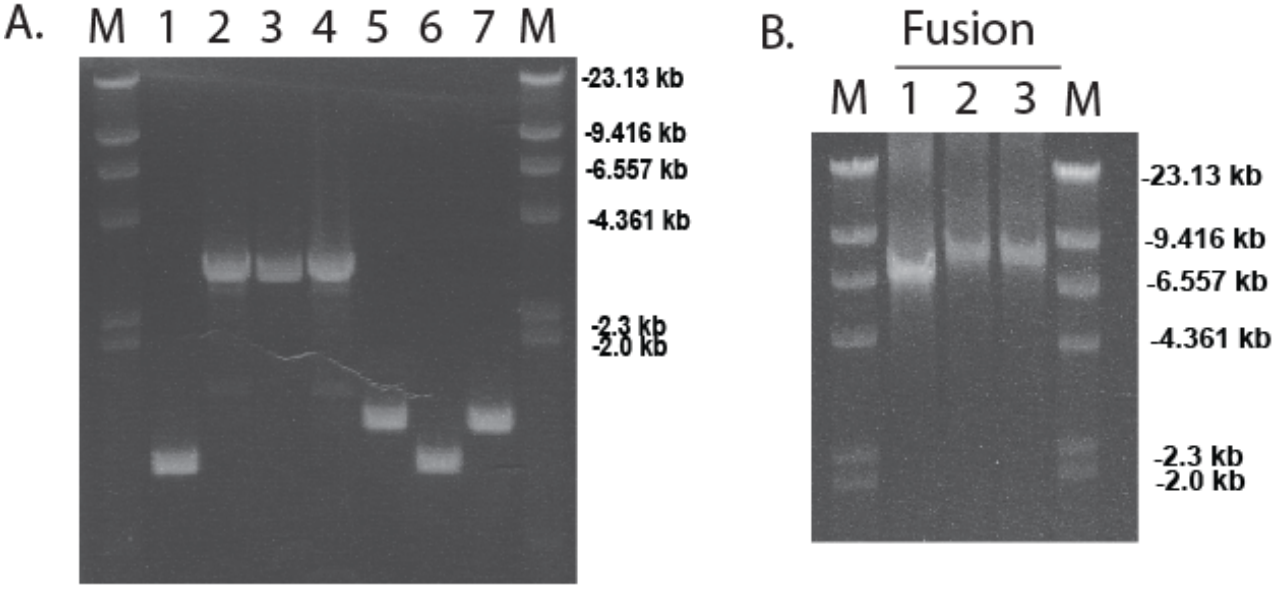



**Figure 2.5: Agarose Gels Confirming the identity of generated pieces 1-7 and fusion PCR reactions.** (A) Amplified DNA pieces generated as mentioned in figure 2.3-2.4 were run on 0.8% Agarose gel to confirm their proper identity. All the pieces run as expected (Expected size is 1kb for piece 1, 2.9kb for 2, 3kb for 3, 2.9kb for 4, 1.2kb for 5, 1kb for 6, and 1.2kb for 7). (B) Fusion PCRs forming the three Nup2 replacement cassettes were also run on 0.8% Agorose gel. The fusion PCR reactions are further explained in figures 2.3-2.4. The fusion cassettes run as expected (Fusion1: 7kb, Fusion 2 and 3: 8kb). The three fusion cassettes generated will be then transformed in SO451 (*pyrG89*) and transformants will be selected on –UU.

# CHAPTER III

# RESULTS

## 3.1 Induction of Nup50 expression under the alcA promotor in *Aspergillus nidulans* does not complement Nup2's function

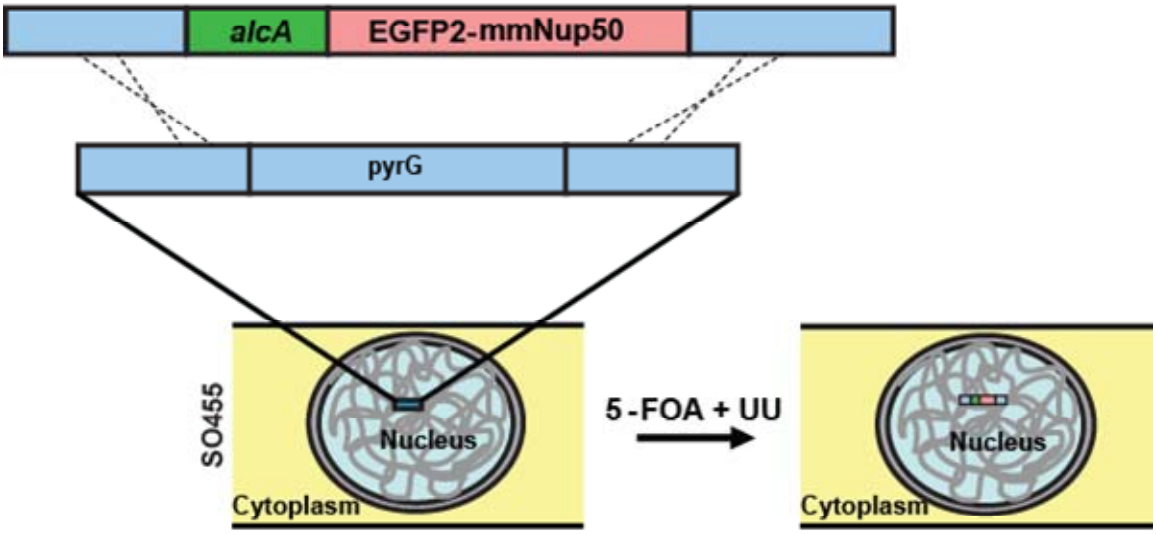
In an attempt to test whether the *Mus musculus*(mm)-Nup50 complements the *Aspergillus nidulans* Nup2, we introduced mmNup50 into the *A. nidulans* genome and expressed it under the alcA inducible promotor. Next, we deleted An-Nup2 in this background. Heterokaryons were identified by streaking conidia from transformants on YAG and YAGUU plates. Conidia from either *Δnup2; alca::EGFP2-mmNup50* heterokaryons or *Δnup2* heterokaryons were then streaked on minimal media dextrose (repressing) and ethanol (inducing) plates lacking Uridine/Uracil. The Δ*nup*2; *alcA::EGFP2-mmNup50* and Δ*nup2* mutant cells grow with similar rates (data not shown). Consequently, this suggests that high levels of mmNup50 may not complement Nup2's function in *Aspergillus nidulans*.

## 3.2 alcA induced Nup50 is unable to locate to either the NPC nor to mitotic chromatin in the presence or absence of endogenous Nup2

To study whether Nup50 can translocate to mitotic chromatin in *A. nidulans* we followed EGFP2-Nup50 in the presence (Figure 3.1B) and absence of endogenous Nup2 (data not shown) throughout the cell cycle. In both cases, Nup50 was nuclear in interphase and dispersed throughout the cell during mitosis. This suggests that the mechanisms by which Nup50 targets the NPC and chromatin are different from the ones utilized by *A. nidulans.* Due to the fact that Nup50 is a much smaller protein than An-Nup2, and that it lacks a domain within Nup2, domain 4, which is responsible to take Nup2 to the pore and to DNA (Markossian, unpublished), we

propose that introducing domain 4 into mmNup50 might tether it to the pore at interphase and mitotic chromatin in *Aspergillus nidulans* and may be able then to complement Nup2's essential function. Experiments are underway to test this hypothesis.

A.


alcA
EGFP2-mmNup50
pyrG
SO455
Nucleus
Cytoplasm
5-FOA + UU
Nucleus
Cytoplasm

B.

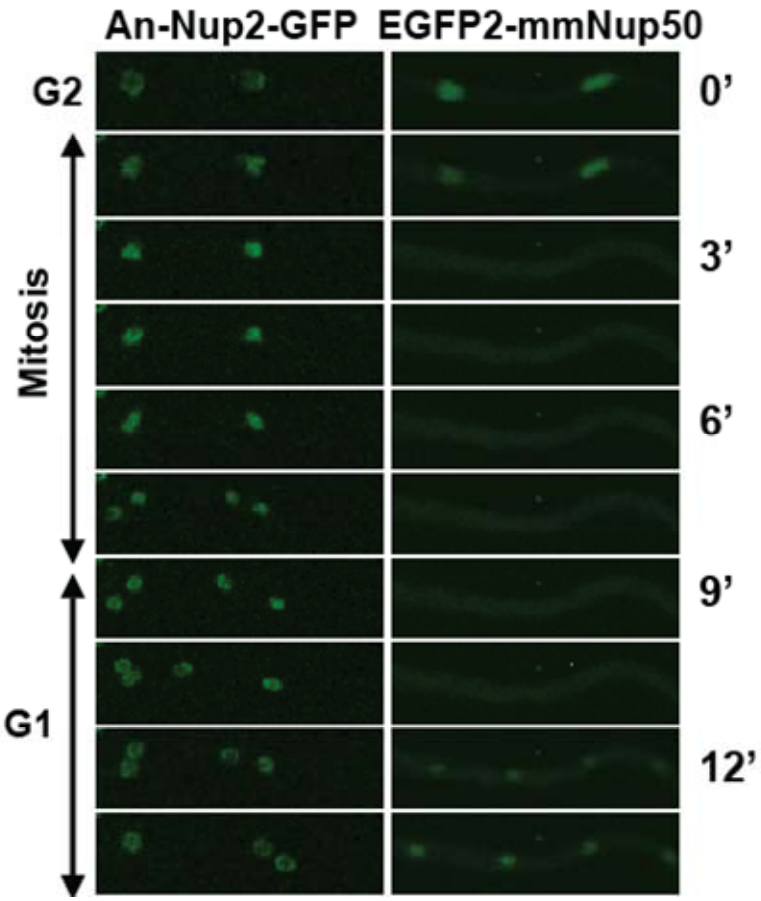

An-Nup2-GFP
EGFP2-mmNup50
G2
Mitosis
G1
0'
3'
6'
9'
12'

**Figure 3.1: mmNup50 locates to the *Aspergillus nidulans* nucleus in interphase and disperses throughout the cell in mitosis. (A)** The *alcA::EGFP2-mmNup50* generated cassette was transformed into the *pyrG* locus of the *A. nidulans* strain SO455. Transformants were counter selected on plates containing 5-Fluoroorotic acid (5-FOA) and Uracil/Uridine to select for the ones that have the *pyrG* gene deleted and the construct integrated at this locus. **(B)** Depicted are still images from time lapse confocal microscopy of living cells carrying either the endogenously tagged An-Nup2-GFP or EGFP2-mmNup50. Nup2 locates to the NPC at interphase and to DNA during mitosis, but its mammalian homolog is nuclear in *A. nidulans* at interphase and disperses throughout the cell during mitosis. Bar, ~ 5μm. This experiment was performed in collaboration with Dr. Sarine Markossian.

# CHAPTER IV

# DISUCCSION AND FUTURE DIRECTION

In *Aspergillus nidulans,* Nup2 moves from nuclear pores to DNA during mitosis (Osmani et al., 2006). Because the mammalian orthologue of Nup2, Nup50 also locates to the vicinity of chromatin during mitosis (Dultz et al., 2008), we thought that Nup2 might have a conserved function at mitotic chromatin. In this work, we attempted to investigate whether Nup50 could complement Nup2's essential role in *A. nidulans*. Our work showed that mammals most likely evolved a distinct mechanism to target Nup2 to either the NPC at interphase or chromatin during mitosis, which is independent of domain 4. Although our attempts to complement An-nup2 with Nup50 have been yet unsuccessful, they provide a good direction for future attempts in asking this question. If successful, this study will provide evidence that the mitotic role of Nup2 is conserved to higher eukaryotes. Consequently, this may reveal some information on the role of Nup50 in open mitosis of higher eukaryotes.

## 4.1 Nup50 is tethered to the mammalian NPC and mitotic chromatin in a manner which is independent of domain 4

Nup 50 has been shown to have analogous dynamic translocation in mitosis to its ortholog Nup2. Similar to Nup2, Nup50 targets to the NPC during interphase and then to chromatin during mitosis. However, when Nup50 was expressed in *A. nidulans*, it neither located to the pore nor to mitotic chromatin. As a result, we have been led to believe that the mechanism in which Nup50 locates onto chromatin during mitosis is distinct from that of Nup2. This result also suggests that

the N-terminally tagging Nup50 with two EGFPs might render the Nup50 protein nonfunctional which might explain why it cannot complement An-Nup2's function.

### 4.2. How to test for complementation in the future

Nup2 targets to nuclear pores and to DNA in distinct ways in *A. nidulans* and mammals. In order to be able to study whether Nup50 really does complement Nup2's essential function, we need to target Nup50 to its proper locations in *A. nidulans*. Correspondingly, we derived the idea to introduce domain 4 into Nup50 to hopefully force it to the pore and to mitotic chromatin. Studies have shown Domain 4 within Nup2 is sufficient to target GFP to NPCs and mitotic chromatin and necessary to locate Nup2 at both places (Markossian, unpublished). Thus, we are currently working on experiments to artificially locate Nup50 to the pore and mitotic chromatin in *Aspergillus nidulans* by tagging it to Domain 4 and asking whether Nup50 will now be able to complement Nup2's essential function. We will also test to see whether the endogenous replacement of Nup2 with Nup50 without Domain 4 complements An-Nup2's function.

## Bibliography

Alber, F., Dokudovskaya, S., Veenhoff, L.M., Zhang, W., Kipper, J., Devos, D., Suprapto, A., Karni-Schmidt, O., Williams, R., Chait, B.T.*, et al.* (2007). Determining the architectures of macromolecular assemblies. Nature *450*, 683-694.

Alberts, B. (2008). Molecular biology of the cell, 5th edn (New York, Garland Science).

Casselton, L., and Zolan, M. (2002). The art and design of genetic screens: filamentous fungi. Nat Rev Genet *3*, 683-697.

D'Angelo, M.A., and Hetzer, M.W. (2008). Structure, dynamics and function of nuclear pore complexes. Trends Cell Biol *18*, 456-466.

De Souza, C.P., Osmani, A.H., Hashmi, S.B., and Osmani, S.A. (2004). Partial nuclear pore complex disassembly during closed mitosis in Aspergillus nidulans. Curr Biol *14*, 1973-1984.

De Souza, C.P., and Osmani, S.A. (2007). Mitosis, not just open or closed. Eukaryot Cell *6*, 1521-1527.

De Souza, C.P., and Osmani, S.A. (2009). Double duty for nuclear proteins--the price of more open forms of mitosis. Trends Genet *25*, 545-554.

Dultz, E., Zanin, E., Wurzenberger, C., Braun, M., Rabut, G., Sironi, L., and Ellenberg, J. (2008). Systematic kinetic analysis of mitotic dis- and reassembly of the nuclear pore in living cells. J Cell Biol *180*, 857-865.

Galagan, A.R.a.J.E. (2008). *Aspergillus nidulans* genome and a comparative analysis of genome evolution in *Aspergillus*. In The Aspergilli : genomics, medical aspects, biotechnology, and research methods, S.A. Osmani, and G.H. Goldman, eds.

Guttinger, S., Laurell, E., and Kutay, U. (2009). Orchestrating nuclear envelope disassembly and reassembly during mitosis. Nat Rev Mol Cell Biol *10*, 178-191.

Laurell, E., Beck, K., Krupina, K., Theerthagiri, G., Bodenmiller, B., Horvath, P., Aebersold, R., Antonin, W., and Kutay, U. (2011). Phosphorylation of Nup98 by Multiple Kinases Is Crucial for NPC Disassembly during Mitotic Entry. Cell *144*, 539-550.

Nayak, T., Szewczyk, E., Oakley, C.E., Osmani, A., Ukil, L., Murray, S.L., Hynes, M.J., Osmani, S.A., and Oakley, B.R. (2006). A versatile and efficient gene-targeting system for Aspergillus nidulans. Genetics *172*, 1557-1566.

Oakley, B.R.a.O., S.A. (1993). Cell-cycle analysis using the filamentous fungus *Aspergillus nidulans*. In The Cell Cycle: A Practical Approach, P.F.a.R. Brooks, ed. (Oxford University Press, USA ).

Osmani, A.H., Davies, J., Liu, H.L., Nile, A., and Osmani, S.A. (2006). Systematic deletion and mitotic localization of the nuclear pore complex proteins of Aspergillus nidulans. Mol Biol Cell *17*, 4946-4961.

Stewart, M. (2007). Molecular mechanism of the nuclear protein import cycle. Nat Rev Mol Cell Biol *8*, 195-208.

Todd, R.B., Davis, M.A., and Hynes, M.J. (2007a). Genetic manipulation of Aspergillus nidulans: heterokaryons and diploids for dominance, complementation and haploidization analyses. Nat Protoc *2*, 822-830.

Todd, R.B., Davis, M.A., and Hynes, M.J. (2007b). Genetic manipulation of Aspergillus nidulans: meiotic progeny for genetic analysis and strain construction. Nat Protoc *2*, 811-821.